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Clusterin expression is upregulated following acute head injury and localises to astrocytes in old head injury

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Short running title: Clusterin expression in head injury

Abstract

There is mounting evidence linking traumatic brain injury (TBI) to neurodegeneration. Clusterin (Apoliporotein J or ApoJ) is a complement inhibitor that appears to have a neuroprotective effect in response to tissue damage and has been reported to be upregulated in Alzheimer's disease. Here we investigated the time course and cellular expression pattern of clusterin in human TBI. Tissue from 32 patients with TBI of varying survival times, (from under 30 minutes to ten months), was examined using immunohistochemistry for clusterin alongside other markers of neurodegeneration and neuroinflammation. TBI cases were compared to ischaemic brain damage, Alzheimer's disease and controls. Double immunofluorescence was carried out in order to examine cellular expression. Clusterin was initially expressed in an axonal location less than 30 minutes following TBI and increased in intensity and the frequency of deposits with increasing survival time up to 24 hours, after which it appeared to reduce in intensity but was still evident several weeks after injury. Clusterin was first evident in astrocytes after 45 minutes, being increasingly seen up to 48 hours but remaining intense in TBI cases with long survival times. Our results suggest clusterin plays a role in modulating the inflammatory response of acute and chronic TBI and that it is a useful marker for TBI, particularly in cases with short survival times. Its prominent accumulation in astrocytes, alongside a mounting inflammatory response and activation of microglial cells supports a potential role in the neurodegenerative changes that occur as a result of TBI.

Key words: head injury, clusterin, neurodegeneration, astrocytes, timing

Introduction

Clusterin, also known as Apolipoprotein J (ApoJ), is a highly conserved ubiquitous glycoprotein expressed in mammalian cells. It is a stress-induced chaperone which is normally secreted, however, in conditions of cellular stress it can be transported to the cytoplasm where it binds to Bax protein and inhibits neuronal apoptosis [1]. Additionally clusterin is a well-known complement inhibitor, binding to C5b-7 component and inhibiting the formation and membrane binding of MAC (membrane attack complex). Expression of clusterin is mainly seen in astrocytes and is also prominent in pyramidal cells of the hippocampus and the Purkinje cell layer of the cerebellum [2]. Several studies have demonstrated that clusterin is involved in the immune response, for example, Interleukins-1B and -2 have both been shown to increase the expression of clusterin in astrocytes [3].

The expression of the Clusterin (*CLU*) gene has been shown to be up-regulated by a multitude of stress and cellular injuries, as well as cellular growth, differentiation and ageing. Gene expression has also been shown to be increased in several disease states, including neurodegeneration, atherosclerosis and cancer [4].

It has been known for a number of years that clusterin expression is increased in brain tissue from patients with Alzheimer's disease (AD) and it was subsequently observed that clusterin can bind amyloid-β (Aβ) peptides and prevent their fibrillization [5]. Clusterin is also involved in the clearance of amyloid-β peptides and fibrils and, by its effect as a complement inhibitor, can suppress the complement activation seen in AD; moreover it appears that the accumulation of misfolded proteins, such as seen in neurodegenerative diseases, can induce clusterin expression [1]. Recently variants within the *CLU* gene were identified as a susceptibility factor for late onset AD by a large genome-wide association study [6]. Indeed, clusterin protein levels are increased in amyloid-based mouse models of AD and early in disease in blood from human patients [7, 8]. In AD post-mortem tissue, clusterin has been shown to be present in neuritic plaques, neuropil threads, cerebrovascular deposits and, to a lesser extent, in diffuse amyloid plaques [9]. A recent study [10] demonstrated that amyloid-β rapidly targets the clusterin protein, causing its intracellular accumulation, with the authors concluding that amyloid-induced neurotoxicity is via the clusterin dependent induction of Dickkopf-1 (Dkk1), which then drives the Wnt-planar cell polarity pathway to increase the expression of genes involved in the neurodegenerative process.

Head injury causes an acute inflammatory response, which can last minutes, days or even months. An immediate severe response occurs in order to minimise tissue damage, which involves activation of the complement system [11] (this may actually also contribute to further "secondary damage"). However, research suggests head injuries can also cause a long-term degenerative process similar to that observed in neurodegenerative diseases [12-16].

Up-regulation of Apolipoproteins (including clusterin) in reactive astrocytes and neurons modulates the inflammatory response found shortly after CNS insults, such as traumatic brain injury (TBI). Previous studies have shown expression of clusterin in animal models of head injury, with an increase in clusterin being seen in the first 2 days after injury and then a return to baseline by 2 weeks which is followed by a second increase lasting up to 6 months [17]. Both clusterin and ApoE were found to co-localise with amyloid-β accumulation in neurons and astrocytes 1-6 months after injury, suggesting clusterin may play a role in the acute response to trauma since clusterin is involved in suppression of complement activation it may have a protective role to prevent secondary damage and then re-emerge long after the initial insult, potentially modulating neurodegenerative changes.

In this study we have investigated the time course and cellular expression of clusterin in human TBI.

Materials and Methods

Post-mortem tissue samples

Brain tissue samples in 10% formalin-fixed, paraffin-embedded tissue blocks were available from the London Neurodegenerative Diseases Brain Bank (King's College London, UK). Consent for autopsy, neuropathological assessment and research were obtained and all studies were carried out under the ethical approval of the tissue bank. Block taking for histological and immunohistochemical studies and neuropathological assessment for neurodegenerative diseases was performed in accordance with standard criteria.

A total of 32 cases of traumatic brain injury were investigated (mean 43±20yr) with varying survival times from the initial injury (see Table 1 for case details). All these cases suffered a closed head injury with no significant intra-cranial haemorrhage. Although cases with significant focal cerebral lesions (such as contusions) were excluded where possible

occasional cases had small thin films of subarchnoid haemorrhage or mild extradural or subdural haematomas (Table 2). Occasional small contusions were noted and a small number of brains revealed at most mild cerebral swelling (Table 2). Nine cases were reported as having a survival of less than 60 minutes; five cases had a survival time of 1-12 hours; three cases had a survival time of 13-24 hours; four cases had a survival time of 25-48 hours; seven cases had a survival time of 8-49 days and four cases showed a survival time of 6-10 months.The causes of death in cases HI1-28 were severe head injury. In addition in cases HI25 and 27 there were additional ischaemic changes contributing (with some small cerebral infarcts in HI27). Case HI29 later died of drowning, cases HI30 and 31 suffered severe epileptic fits, and case HI32 died from asphyxia due to hanging.

Four cases of Alzheimer's disease were also investigated (mean 84±8yr). These cases had both a clinical diagnosis of Alzheimer's disease and clear pathological evidence of Alzheimer's disease with no other pathology. These cases were used as a positive control for the presence of clusterin (see Table 3).

Three cases of ischaemic damage to the brain were selected from patients with hypovolemic shock after a stab wound, intestinal bleeding and cardiac arrest (mean 44±17yr, Table 3). Detailed post mortem examination was carried out on these cases and it was demonstrated that there was no other pathological process or disease affecting the body or the brain.

Five control cases were also selected (mean 38±13yr, Table 3). All these cases had previously been comprehensively examined by histological examination and immunohistochemistry and showed no evidence of any pathological process.

In all the studied cases, the following regions were examined: frontal lobe, temporal lobe, hippocampus, corpus callosum, basal ganglia, midbrain and pons. Blocks were selected distant from focal pathology such as small contusions and haemorrhage.

Immunohistochemistry:

The immunohistochemistry technique for clusterin was carried out as per previously published protocols [18]. In brief, sections of 7µm thickness were cut from the paraffin-embedded tissue blocks, deparaffinised in xylene, endogenous peroxidase blocked by immersion in 2.5% H_2O_2 in methanol and immunohistochemistry performed. To enhance antigen retrieval, sections were kept in citrate buffer for 10 minutes following microwave

treatment. After blocking in normal swine serum (DAKO, Cambridgeshire, UK), clusterin antibody (AbCam, ab69644) was applied at 1:500 overnight at 4°C.

Following washes, sections were incubated with biotinylated secondary antibody (DAKO), followed by avidin:biotinylated enzyme complex (Vectastain Elite ABC kit, Vector Laboratories, Peterborough, UK). Finally sections were incubated for 5–10 min with 0.5 mg/mL 3,3′-diaminobenzidine chromogen (Sigma-Aldrich Company Ltd, Dorset UK) in Tris-buffered saline (pH 7.6) containing 0.05% H_2O_2. Sections were counterstained with Harris' haematoxylin and immunostaining analysed using a Leica microscope (Leica, Wetzlar, Germany).

Additionally, immunohistochemistry with the following antibodies was carried out during the diagnostic assessment of all cases examined (except for GFAP, S100, and C5b-9, which were applied to cases HI12, 13, 15, 20, 22, 24, 26, 27, 30, 31 C4 and C5) using the Leica BONDMAX™ (Leica Biosystems, Wetzlar, Germany): Aβ (clone 4G8; 1:1000, Chemicon, Temecula, CA), βAPP (clone 22C11; 1/10000, Chemicon, Temecula, CA), p62 (1/100, BD Biosciences, Erembodegem, Belgium), phosphorylated tau (clone AT-8; 1:500, Autogen Bioclear UK Ltd, Wiltshire, UK), phosphorylated pTDP-43 (pS409/410-2); 1:1500, Cosmo Bio Ltd, Tokyo, Japan), GFAP (Z0334; 1:1000, Dako, Glostrup, Denmark) Complement (MAC)- C5b-9(Clone aE11; 1:50 , Dako), S100(Z0311; 1:1500, Dako) CD68 (clone PGM1; 1:50, Dako).

Double Immunofluorescence:

To identify the cell types expressing clusterin and to investigate potential co-localisation of clusterin with βAPP, double immunofluorescence was carried out on a sub-set of cases (three cases of AD and six cases of head injury (three acute, three old)) (labelled § in Tables 1 and 3). Healthy control tissue was also examined. The details of the antibodies and dilutions are given in Table 4.

The 7µm sections were cut from formalin fixed paraffin embedded blocks, dewaxed in xylene and dehydrated in 99% industrial methylated spirit. Sections were then pretreated by microwaving in citrate buffer and blocked using normal goat serum (1:10 for 45min). Primary antibodies against clusterin, βAPP, GFAP and markers of activated microglia were then applied (see Table 4 for full details) and sections incubated at 37°C for 1hr or at 4°C overnight. Sections were washed and secondary Alexa Fluor antibody (goat anti-mouse and

goat anti-rabbit, Invitrogen, Paisley, UK) applied for 45 min (in dark). Autofluorescence was quenched by incubating the sections in Sudan black for 10min followed by numerous washes in phosphate buffered saline before coverslip mounting using Vectashield hard set media with DAPI. Sections were visualised using a fluorescent microscope (Zeiss Axiovert S 100, Gottingen, Germany) and images captured using ImagePro Express (v6).

Results

Expression of clusterin in Alzheimer's disease

These cases were confirmed to demonstrate Alzheimer's disease pathology, showing intense immunoreactivity with abnormally phosphorylated tau in plaques, tangles and neuropil threads. There was intense immunoreactivity of Aβ in the cortex with all cases showing a variable degree of deposition in the meningeal and some cortical blood vessels, consistent with amyloid angiopathy. The p62 was positive in some tangles and plaques but pTDP-43 staining was negative in all cases. βAPP staining showed a variable degree of immunoreactivity in the neuritic plaques and scattered positivity in the cortex (See Table 3 for summary of pathology).

We were also able to reproduce previously reported expression of clusterin in Alzheimer's disease brain tissue. The clusterin was seen densely aggregated in neuritic plaques and immuno-positive neurites were observed (Figure 1A,B). There was granular positivity of varying intensity in the glial cells of the white matter (Figure 1C).

Expression of clusterin in head injury

Clusterin immunohistochemistry was performed on 32 cases of head injury including 28 acute head injury (defined as a survival period of 25min up to 46 days post injury (mean age 46±20yr) and four cases of old head injury with a survival time of 6-10 months (mean age 27±14yr).

Clusterin expression was demonstrated in all the cases of head injury but in varying densities and cellular location (See Table 1 for summary of pathology). Clusterin immunoreactivity was seen predominantly in the white matter and white matter tracts, such as the corpus callosum and internal capsule, and less often in the cerebral cortex and grey matter.

In the cases of acute head injury with a short survival time, clusterin was seen as occasional small intra-axonal varicosities or globules, most likely representing sites of axonal

dysfunction or disruption. It was detected in cases with a survival time of less than 30 minutes (Figure 2A) and appeared to increase in parallel with increased survival time; being easily recognised after 60 minutes where more than just occasional deposits were identified. The deposits increased in frequency and intensity forming globules, filamentous structures of thick and thin filaments in cases with survival of more than one hour and continued to increase in intensity up to approximately 24 hours (Figure 2B-E); after which the intensity of staining was slightly reduced but still with many globules or thickened filaments (Figure 2 F, G and 3 A-C).

In the cases with a survival time of 46 and 49 days the axonal intensity of clusterin staining was observed to be much less, with a lower density and fainter staining, and in the latter case only occasional deposits could be found (Figure 3 D). After this time this pattern was no longer seen.

The clusterin immunoreactivity in the axonal location was similar to the βAPP staining. However, clusterin was evident in cases with a survival time of less than 30 minutes, whereas βAPP was only identified in this study following survival of 45 minutes or longer. The other difference was that clusterin showed granular immunoreactivity in very occcasional glial cells after 45 minutes survival. As time progressed there was an increase in the number of glial cells staining and in the intensity of staining so as to be more obvious at 48 hours (Figure 2 H,I) and clusterin continued to be moderately over-expressed in survival times of several weeks to several months (Figures 3 A-D insets and 4); in these later cases, the clusterin displayed focal staining, present as cytoplasmic granular deposits in glial cells in the corpus callosum and white matter of the cerebral hemispheres and internal capsule. This appeared to correlate with the GFAP positive glial cells, thus strongly implying that astrocytes were the labelled cells (and also often also applied to strongly labelled S100 positive cells) (Figure 5). The smaller oligodendrocytes very occasionally showed very mild positivity but the vast majority appeared negative. However, there was no significant clusterin deposition in the cerebral cortex or hippocampus. The suggested time lines for clusterin immuno-expression is illustrated in Figure 6.

Immunohistochemistry for Tau, Aβ, pTDP-43 and p62 was negative in all cases of recent and old head injury (Table 1). The C5b-9 complement was also negative in all cases, however, despite the use of pretreatment (and confirmed positivity in controls) this may have been a reflection of prolonged fixation of the brains in formalin.

Clusterin expression in ischaemia and contusions

We investigated three cases of recent brain ischaemia due to various causes and with survival times of between 5-24 hours. These cases showed typical appearances of ischaemia, with red shrunken neurones in the cortical deep white matter, hippocampus and Purkinje cells of cerebellum. The clusterin showed clear overexpression in the ischaemic neurones and was deposited as ill-defined areas with granular and fine filamentous pattern similar to those seen in staining with βAPP (Figure 7A). However, this expression appeared to be different from the pattern of clusterin and βAPP deposition seen in cases of head injury where the deposits were seen as well-defined globules and thickened filaments with no granular background

In all control cases the staining for clusterin was negative (Figure 7B), as was βAPP, tau, Aβ, pTDP43 and p62. CD68 showed an expected increase in the activated microglial cells with increased survival time after 48 hours and continued to be intensely over expressed in cases of old head injury of 6-10 months (Table 1). In these cases there were also many small accumulations of the glial cells consistent with microglial nodules. The main purpose of the study was to concentrate on the changes applicable to more diffuse elements of TBI cases. Therefore the cases were deliberately chosen in order to reduce the contribution of focal elements of trauma. By the very nature of the injury, however, it was often not possible to totally eliminate focal damage such as contusions but there was an effort to concentrate on cases with at most only mild contusions (Table 2). The blocks from the test cases were taken far from these areas and any other likely focal pathology. When examined separately, the clusterin in the area of the actual contusions did show immunopositivity with expression in astrocytes and some neurones (images not shown). There were also angulated eosinophilic neurones in these regions and some appearing apoptotic.

All other markers for tau, pTDP-43, Aβ and p62 were negative in cases of acute and old head injury

Co- expression of clusterin with relation to βAPP

Double fluorescence microscopy was performed on sections of corpus callosum from three head injury cases and three AD cases (labelled § in Table 1 & 2). All 3 cases of TBI demonstrated adjacent expression but no co-localisation between clusterin and βAPP (survival times of 24-36 hr). The head injury case with 1-12 hr survival time illustrated a

mild scattering of dots of clusterin and β-APP. In contrast, at 24-36 hr there was a significant increase in the deposition of both clusterin and βAPP and the deposition had a beaded like appearance (Figure 8A). In the AD case adjacent expression of clusterin with βAPP was observed in plaques, however, overall, there was a greater amount of βAPP staining in the plaques than was observed for clusterin (Figure 8B).

Identification of cell-types expressing clusterin

Using double immunofluorescence cell specific expression was examined in a subset of cases using antibodies against astrocytes and activated microglia, alongside those for clusterin.

Clusterin was not expressed in astrocytes in cases of acute head injury with survival of less than 30 minutes, but was evident in astrocytes in old head injury (Figure 8C). In cases of old head injury (at least 6 months survival after initial injury) astrocytes had a typical reactive morphology.

Using a CD68 antibody as a marker for microglial cells associated with phagocytosis, microglia were observed alongside clusterin in head injury cases (Figure 9 A, B). However, there was no co-localisation of clusterin with CD68. Old head injury cases showed similar findings to recent head injuries; however, there was somewhat more microglial activation present in old head injuries (Figure 9 A,B). In AD sections there were numerous microglia evident together with clusterin, particularly near plaques, however, the microglia did not appear to be expressing clusterin (images not shown).

Immunofluorescence was also performed using an HLA-DR clone CR3/43, a marker for activated microglia (images not shown). Findings were similar to the CD68; for head injury cases both microglia and clusterin were present, in one case there appeared to be some co-localisation, which was not observed in either of another two recent head injury cases. In the old head injury cases, larger densities of microglia were activated than in acute head injury, but there was no co-localisation evident. In the AD cases activated microglia were seen, as were clusterin deposits, but again there was no co-localisation seen.

Discussion

In this study we have demonstrated an over-expression of clusterin in TBI in comparison to control brain tissue. To our knowledge this has not been demonstrated in human post mortem tissue previously and is in keeping with experimental data obtained in animals [17, 19, 20].

We have shown that clusterin is over-expressed very soon after the onset of TBI, suggesting that it plays a role in the response to blunt trauma and that it may assist in remodelling and modulation of the inflammatory response following this form of brain injury. There was staining of clusterin adjacent to βAPP when examined by double immunofluorescence in white matter - which is consistent with overexpression at the site of axonal injury and dysfunction.

Clusterin was observed deposited in the white matter of brains from patients who died from head injury as early as 25 minutes after the initial injury. The exact time of death in this type of cases is often difficult to ascertain, being dependent on paramedic evaluation. Therefore, it was quite possible that death could have occurred even earlier than 25 minutes and that clusterin is deposited extremely rapidly after TBI. Clusterin would appear to be an even more sensitive marker for early axonal injury than βAPP [21]; an important observation for medico-legal practice and may assist in the diagnosis and timing of TBI in patients who died quickly after head trauma

Like βAPP [22, 23], clusterin was also overexpressed in the white matter of brains from patients who died from ischaemia without trauma. Indeed clusterin has been seen to be overexpressed in association with global ischaemia, gliomas, white matter diseases, epileptic foci and both chemical and traumatic brain and spinal cord injuries [5, 17, 24-27]. Therefore, clusterin can be considered as a sensitive marker for axonal damage but not specific as to its cause. It may even be involved with axonal regeneration in the peripheral nervous system [28]

We found persistent and increasing immunoexpression of clusterin in the axons, corresponding to increasing survival times of the patients after head injury. It appeared to reach a maximum expression in 24 hours after which the intensity of clusterin deposits appeared to decrease, forming paler-staining and granular globules and filaments and became much sparser such that only a few granular deposits were seen in the brains of patients who survived 49 days after head injury. However, this observation has to be

considered with caution; firstly because not all the cases had the same degree of head injury and hence the amount of clusterin deposition may vary and secondly because the assessment was carried out using a semi-quantitative method

Interestingly, compared with intra-axonal clusterin deposition in the white matter, we found that the deposition of clusterin in occasional glial cells was seen in brains of patients who died 45 minutes after injury and increased in frequency and intensity (as cytoplasmic accumulation) after 24 hours before reaching an apparent plateau at 48 hours. Since there was co-expression of clusterin and GFAP the vast majority of these glial cells were likely to be astrocytes similar to findings following cord injury[25]. These findings are consistent with the function of clusterin as a chaperone protein [29]. The clusterin deposits in the astrocytes persisted for several months after the head trauma and were demonstrated intensely in patients who survived 9 and 10 months after head injury. Clusterin has a short half-life and it may be that in the first acute phase there is local intra-axonal clusterin protein synthesis performed as a consequence of axonal shearing (and the local production of stress proteins) and perhaps in an attempt at axonal regeneration which has been shown to occur in peripheral nerves[28, 30, 31]. After this regeneration has been partially achieved or prevented this aspect of clusterin production is reduced and replaced by astrocytic clusterin protection perhaps to aid protection against inflammatory mediators and toxic proteins[25, 30, 31]. This different location and timing of expression of clusterin, first at the site of axonal injury soon after head trauma and later accumulating in astrocytes in older head injury, could be seen to be a two track response and would be similar but not identical to the biphasic increase in protein concentration as reported in experimental head injury. Iwata *et al.,* demonstrated an increase in protein deposition and number of cells immunoreactive for clusterin, initially peaking in 2 days, returning to baseline level by 2 weeks then gradually increasing throughout 6 months post trauma [17]. Our study did show a decrease in the axonal immunoexpression of clusterin to a baseline level between 6 weeks and 6 months, but the astroglial expression once seen was maintained even after 6 months. It could be noted that there were no cases with a survival of between 6 weeks and 6 months in our cohort, and at least theoretically there may have been a decrease and subsequent recovery of astrocytic expression within this time period but it would appear unlikely. The findings also differ from the rat model described in that Iwata *et al*. described accumulation of Aβ alongside clusterin (between 1-6 months), and this was not present in our study [17]. This gradual increase of clusterin in astrocytes in our study occurred with a mounting long-term post-

traumatic neuro-inflammatory process. The latter was clearly demonstrated in our post-mortem tissue, with an increase in the number of microglia cells (as demonstrated with CD68 antibody) after 24 hours, which became more obvious after 48 hours and persisted to a high level of expression even in patients surviving 10 months after head injury. These findings further support the evidence that clusterin may play an active role in modulating the neuroinflammatory response and possibly has a protective role even several months after head injury. Previous research has demonstrated a long term neuroinflammatory response by microglia and infiltrating macrophages after head injury [13]; these cells are the source of cytokines and this may in turn incite more activation of astrocytes, and increase S100 protein and then induce further activation of microglial cells hence enabling a sustained inflammatory response.

It is possible that the accumulated clusterin in the white matter astrocytes may not only have a role in modulating the increasing post trauma neuroinflammation but may also represent a protective process (or at least a better reparative process) by lipoproteins after head injury. Imhof *et al*., proposed that sustained increase of clusterin expression in astrocytes improved structural recovery after ischaemic insults since the restoration process was clearly slower and less successful in clusterin knock down mice compared to that seen in wild type [19]. There is also supportive evidence from patients with clusterin (*CLU*) gene mutations that the protein is important in maintaining white matter structural integrity[32]. As we have confirmed in this study, clusterin was typically present in neuritic plaques and neuropil threads but rarely in primitive plaques and neurofibrillary tangles [33]. It has been reported that clusterin can target and act as a chaperone for amyloid peptides and mediate their clearance but later enhances the formation of the plaques when the amyloid is fibrillized and aggregated. Interestingly, the absence of astrocytic clusterin in association with Alzheimer disease plaques may be due to some mutual regulatory process[34, 35]. There appears a similar lack of astrocytic clusterin expression in the vicinity of prion protein plaques in experimental prion disease[36]. Clusterin can inhibit the complement activation and mask the misfolded protein to prevent excessive inflammation and neuronal death, although the findings in the injured nervous system are sometimes contradictory as exemplified by our findings (allowing for the caveat already stated above) [1, 37, 38]. It is therefore possible that clusterin may play a protective role after trauma by binding to Aβ and suppressing its accumulation. This process may explain the lack of any Aβ accumulation in our head injury cases despite the presence of a high level of βAPP deposition.

There are several reports of increasing risk of dementia and Alzheimer's disease after TBI [14, 39]. However, other epidemiological studies have failed to confirm such association [40]. It appears that there are many unanswered questions resulting from these largely retrospective and complex observational studies about the link between a single TBI and dementia and its actual prevalence. The pathology and clinical manifestation of chronic traumatic encephalopathy (CTE) and its relation to repeated minor trauma is well established, e.g. that seen in boxers, military veterans and American football players. Pathologically it is characterised by abnormal phosphorylated tau deposition at the depth of sulci and around blood vessels in early stages, involving other regions in the brain in more advance stages in addition to TDP-43 pathology [41-43]. On the other hand the "single episode TBI" associated dementia is often thought to be Alzheimer's type of dementia [44], however, this is not absolutely proven and the association appears to be more complex

In contrast to the accumulation of clusterin in the astrocytes of the white matter in our cases of old head injury, we did not find any significant accumulation in the neurons. The stains for tau, pTDP-43, Aβ and p62 were negative in all our cases of head injury, including those with several months' survival; which may exclude the possibility of early Alzheimer's disease or TDP-43 related pathology. These findings suggest either that there is no, or only a minor indirect or as yet unexplained complex role of clusterin in post-traumatic Aβ plaques and Alzheimer's disease changes. Therefore, the question as to how diffuse white matter axonal damage could later lead to a neurodegenerative disease defined predominantly by cortical neuronal pathology remains unanswered. The investigation of clusterin immunoexpression in a cohort of well-established chronic traumatic encephalopathy cases (following repeated low level head injury) compared with Alzheimer's disease cases (with and without a pre-existing history of TBI) will therefore form the basis of further studies.

In summary, this study not only reveals axonal clusterin expression to be an early response to head injury but also indicates that the protein continues to be expressed in astrocytes several months after the trauma. There is evidence to suggest that clusterin is involved in modulating the inflammatory response. Understanding the role clusterin plays in the pathology of TBI could lead to possible therapeutic interventions. Further studies are needed to authenticate these findings and to investigate the possible relationship between

clusterin expression in cases of repeated mild head injury, old head injury of several years' survival and neurodegenerative conditions such as chronic traumatic encephalopathy.

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Competing interest

The authors declare they have no conflict of interest.

Reference List

1. Nuutinen T, Suuronen T, Kauppinen A, Salminen A. Clusterin: a forgotten player in Alzheimer's disease. *Brain Res Rev* 2009:**61**: 89-104.

2. Pasinetti GM, Johnson SA, Oda T, Rozovsky I, Finch CE. Clusterin (SGP-2): a multifunctional glycoprotein with regional expression in astrocytes and neurons of the adult rat brain. *J Comp Neurol* 1994:**339(3)**: 387-400.

3. Zwain IH, Grima J, Cheng CY. Regulation of clusterin secretion and mRNA expression in astrocytes by cytokines. *Mol Cell Neurosci* 1994:**5(3)**: 229-237.

4. Rosenberg ME, Silkensen J. Clusterin: physiologic and pathophysiologic considerations. *Int J Biochem Cell Biol* 1995:**27(7)**: 633-645.

5. May PC, Lampert-Etchells M, Johnson SA, Poirier J, Masters JN, Finch CE. Dynamics of gene expression for a hippocampal glycoprotein elevated in Alzheimer's disease and in response to experimental lesions in rat. *Neuron* 1990:**5(6)**: 831-839.

6. Harold D, Abraham R, Hollingworth P *et al.* Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. *Nat Genet* 2009:**41(10)**: 1088-1093.

7. Thambisetty M, Simmons A, Velayudhan L *et al.* Association of plasma clusterin concentration with severity, pathology, and progression in Alzheimer disease. *Arch Gen Psychiatry* 2010:**67(7)**: 739-748.

8. Schrijvers EM, Koudstaal PJ, Hofman A, Breteler MM. Plasma clusterin and the risk of Alzheimer disease. *JAMA* 2011:**305(13)**: 1322-1326.

9. Giannakopoulos P, Kovari E, French LE, Viard I, Hof PR, Bouras C. Possible neuroprotective role of clusterin in Alzheimer's disease: a quantitative immunocytochemical study. *Acta Neuropathol* 1998:**95(4)**: 387-394.

10. Killick R, Ribe EM, Al-Shawi R *et al.* Clusterin regulates beta-amyloid toxicity via Dickkopf-1-driven induction of the wnt-PCP-JNK pathway. *Mol Psychiatry* 2014:**19(1)**: 88-98.

11. Bellander BM, Singhrao SK, Ohlsson M, Mattsson P, Svensson M. Complement activation in the human brain after traumatic head injury. *J Neurotrauma* 2001:**18(12)**: 1295-1311.

12. Roberts GW, Gentleman SM, Lynch A, Murray L, Landon M, Graham DI. Beta amyloid protein deposition in the brain after severe head injury: implications for the pathogenesis of Alzheimer's disease. *J Neurol Neurosurg Psychiatry* 1994:**57(4)**: 419-425.

13. Smith C, Gentleman SM, Leclercq PD *et al.* The neuroinflammatory response in humans after traumatic brain injury. *Neuropathol Appl Neurobiol* 2013:**39(6)**: 654-666.

14. Guo Z, Cupples LA, Kurz A *et al.* Head injury and the risk of AD in the MIRAGE study. *Neurology* 2000:**54(6)**: 1316-1323.

15. Millar K, Nicoll JA, Thornhill S, Murray GD, Teasdale GM. Long term neuropsychological outcome after head injury: relation to APOE genotype. *J Neurol Neurosurg Psychiatry* 2003:**74(8)**: 1047-1052.

16. Chauhan NB. Chronic neurodegenerative consequences of traumatic brain injury. *Restor Neurol Neurosci* 2014:**32(2)**: 337-365.

17. Iwata A, Browne KD, Chen XH, Yuguchi T, Smith DH. Traumatic brain injury induces biphasic upregulation of ApoE and ApoJ protein in rats. *J Neurosci Res* 2005:**82(1)**: 103-114.

18. Maekawa S, Leigh PN, King A *et al.* TDP-43 is consistently co-localized with ubiquitinated inclusions in sporadic and Guam amyotrophic lateral sclerosis but not in familial amyotrophic lateral sclerosis with and without SOD1 mutations. *Neuropathology* 2009:**29**: 672-683.

19. Imhof A, Charnay Y, Vallet PG *et al.* Sustained astrocytic clusterin expression improves remodeling after brain ischemia. *Neurobiol Dis* 2006:**22(2)**: 274-283.

20. Strocchi P, Rauzi F, Cevolani D. Neuronal loss up-regulates clusterin mRNA in living neurons and glial cells in the rat brain. *Neuroreport* 1999:**10(8)**: 1789-1792.

21. Hortobagyi T, Wise S, Hunt N *et al.* Traumatic axonal damage in the brain can be detected using beta-APP immunohistochemistry within 35 min after head injury to human adults. *Neuropathol Appl Neurobiol* 2007:**33(2)**: 226-237.

22. Bramlett HM, Dietrich WD. Pathophysiology of cerebral ischemia and brain trauma: similarities and differences. *J Cereb Blood Flow Metab* 2004:**24(2)**: 133-150.

23. Lambri M, Djurovic V, Kibble M, Cairns N, Al-Sarraj S. Specificity and sensitivity of betaAPP in head injury. *Clin Neuropathol* 2001:**20(6)**: 263-271.

24. Han BH, DeMattos RB, Dugan LL *et al.* Clusterin contributes to caspase-3-independent brain injury following neonatal hypoxia-ischemia. *Nat Med* 2001:**7(3)**: 338-343.

25. Klimaschewski L, Obermuller N, Witzgall R. Regulation of clusterin expression following spinal cord injury. *Cell Tissue Res* 2001:**306(2)**: 209-216.

26. Wiggins AK, Shen PJ, Gundlach AL. Delayed, but prolonged increases in astrocytic clusterin (ApoJ) mRNA expression following acute cortical spreading depression in the rat: evidence for a role of clusterin in ischemic tolerance. *Brain Res Mol Brain Res* 2003:**114(1)**: 20-30.

27. Craggs L, Taylor J, Slade JY *et al.* Clusterin/Apolipoprotein J immunoreactivity is associated with white matter damage in cerebral small vessel diseases. *Neuropathol Appl Neurobiol* 2016:**42**: 194-209.

28. Wright MC, Mi R, Connor E *et al.* Novel roles for osteopontin and clusterin in peripheral motor and sensory axon regeneration. *J Neurosci* 2014:**34**: 1689-1700.

29. Nizard P, Tetley S, Le DY *et al.* Stress-induced retrotranslocation of clusterin/ApoJ into the cytosol. *Traffic* 2007:**8(5)**: 554-565.

30. Rizzi F, Caccamo AE, Belloni L, Bettuzzi S. Clusterin is a short half-life, poly-ubiquitinated protein, which controls the fate of prostate cancer cells. *J Cell Physiol* 2009:**219**: 314-323.

31. Baleriola J, Hengst U. Targeting axonal protein synthesis in neuroregeneration and degeneration. *Neurotherapeutics* 2015:**12**: 57-65.

32. Braskie MN, Jahanshad N, Stein JL *et al.* Common Alzheimer's disease risk variant within the CLU gene affects white matter microstructure in young adults. *J Neurosci* 2011:**31**: 6764-6770.

33. Calero M, Rostagno A, Matsubara E, Zlokovic B, Frangione B, Ghiso J. Apolipoprotein J (clusterin) and Alzheimer's disease. *Microsc Res Tech* 2000:**50(4)**: 305-315.

34. Nuutinen T, Huuskonen J, Suuronen T, Ojala J, Miettinen R, Salminen A. Amyloid-beta 1-42 induced endocytosis and clusterin/apoJ protein accumulation in cultured human astrocytes. *Neurochem Int* 2007:**50**: 540-547.

35. Mulder SD, Nielsen HM, Blankenstein MA, Eikelenboom P, Veerhuis R. Apolipoproteins E and J interfere with amyloid-beta uptake by primary human astrocytes and microglia in vitro. *Glia* 2014:**62**: 493-503.

36. Sasaki K, Doh-ura K, Ironside JW, Iwaki T. Increased clusterin (apolipoprotein J) expression in human and mouse brains infected with transmissible spongiform encephalopathies. *Acta Neuropathol* 2002:**103**: 199-208.

37. Tao R, Aldskogius H. Glial cell responses, complement and apolipoprotein J expression following axon injury in the neonatal rat. *J Neurocytol* 1999:**28**: 559-570.

38. Tornqvist E, Liu L, Aldskogius H, Holst HV, Svensson M. Complement and clusterin in the injured nervous system. *Neurobiol Aging* 1996:**17**: 695-705.

39. Mayeux R, Ottman R, Tang MX *et al.* Genetic susceptibility and head injury as risk factors for Alzheimer's disease among community-dwelling elderly persons and their first-degree relatives. *Ann Neurol* 1993:**33(5)**: 494-501.

40. Molgaard CA, Stanford EP, Morton DJ, Ryden LA, Schubert KR, Golbeck AL. Epidemiology of head trauma and neurocognitive impairment in a multi-ethnic population. *Neuroepidemiology* 1990:**9(5)**: 233-242.

41. McKee AC, Stein TD, Kiernan PT, Alvarez VE. The neuropathology of chronic traumatic encephalopathy. *Brain Pathol* 2015:**25(3)**: 350-364.

42. McKee AC, Cantu RC, Nowinski CJ *et al.* Chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. *J Neuropathol Exp Neurol* 2009:**68**: 709-735.

43. King A, Sweeney F, Bodi I, Troakes C, Maekawa S, Al-Sarraj S. Abnormal TDP-43 expression is identified in the neocortex in cases of dementia pugilistica, but is mainly confined to the limbic system when identified in high and moderate stages of Alzheimer's disease. *Neuropathology* 2010:**30**: 408-419.

44. Smith DH, Johnson VE, Stewart W. Chronic neuropathologies of single and repetitive TBI: substrates of dementia? *Nat Rev Neurol* 2013:**9(4)**: 211-221.

Legends to Figures

Figure 1: Clusterin expression in Alzheimer's disease cerebral tissue. Immunohistochemistry demonstrates Clusterin within neuritic plaques (A), neuropil threads (arrowed - B) and glial cells (arrowed - C). Scale bar represents 20μm.

Figure 2: Clusterin expression shown in traumatic brain injury (TBI) cases with increasing time of survival from initial head injury. Clusterin is first seen to deposit as axonal globules, becoming most visible after several hours as globules and filaments. After 24 hours filaments are still numerous but intensity lessens. Glial expression becomes more prominent as survival time increases (up to 48 hours). (panels H & I show glial expression), insets show glial cells at higher magnification.. Scale bar represents 20μm (A-C, F, H, I) and 50μm (D, E, G) and 15μm (H inset and I inset).

Figure 3: Clusterin expression demonstrated in TBI cases with a survival time of several days. Inset panels show glial expression. Scale bar represents 20μm (main images) and 50μm (insets).

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Figure 6: Schematic diagram illustrating the time line after survival from head injury and the relative immuno-expression of clusterin in axons (A) and astrocytic cells (B).

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Figure 9: A and B: There is no co-localisation of clusterin (red), and microglia (CD68 - green) in any of the cases, however, cases of old head injury (A) showed somewhat increased microglial activation compared to acute head injury (B). Scale bar represents 30μm.

Case	Age/ sex	Survival period	Clusterin		βAPP		CD68*	Tau	Aβ	TDP43	p62
			Axons	Glial	Axons	Glial					
HI1 §	34 M	25 min	+	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
HI2 §	30 M	30 min	+	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
HI3	? F	< 30 min	+	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
HI4	30 M	< 30 min	+	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
HI5 §	41 M	45 min	++	+	+	-ve	-ve	-ve	-ve	-ve	-ve
HI6	30 M	50 min	+	+	+	-ve	-ve	-ve	-ve	-ve	-ve
HI7	43 F	< 60 min	+	+	+	-ve	-ve	-ve	-ve	-ve	-ve
HI8	80 M	< 60 min	+	+	+	-ve	-ve	-ve	-ve	-ve	-ve
HI9	25 M	< 60 min	++	+	++	-ve	-ve	-ve	-ve	-ve	-ve
HI10	74 M	3-5 h	+++	+	+++	-ve	-ve	-ve	-ve	-ve	-ve
HI11	83 F	4 h	+++	+	+++	-ve	-ve	-ve	-ve	-ve	-ve
HI12	52 F	6 h	++++	++	++++	-ve	-ve	-ve	-ve	-ve	-ve
HI13	24 M	9 h	++++	++	++++	-ve	-ve	-ve	-ve	-ve	-ve
HI14	44 M	10 h	++++	++	++++	-ve	-ve	-ve	-ve	-ve	-ve
HI15	50 M	16 h	+++++	++	+++++	-ve	-ve	-ve	-ve	-ve	-ve
HI16	53 M	21 h	+++++	++	+++++	-ve	-ve	-ve	-ve	-ve	-ve
HI17	21 M	24 h	+++++	++	+++++	-ve	+	-ve	-ve	-ve	-ve
HI18	43 M	39 h	+++	+++	+++	-ve	+	-ve	-ve	-ve	-ve
HI19	60 M	40 h	+++	+++	+++	-ve	++	-ve	-ve	-ve	-ve
HI20	29 M	48 h	+++	+++	+++	-ve	++	-ve	-ve	-ve	-ve
HI21	25 F	48 h	+++	+++	+++	-ve	++	-ve	-ve	-ve	-ve
HI22	72 M	8 d	+++	++	++	-ve	+++	-ve	-ve	-ve	-ve
HI23	61 M	14 d	+++	+++	++	-ve	++++	-ve	-ve	-ve	-ve
HI24	55 M	14 d	+++	+++	++	-ve	++++	-ve	-ve	-ve	-ve
HI25 §	21 M	22 d	+++	+++	++	-ve	++++	-ve	-ve	-ve	-ve
HI26	16 M	27 d	+++	+++	++	-ve	++++	-ve	-ve	-ve	-ve
HI27	59 M	46 d	++	+++	+	-ve	++++	-ve	-ve	-ve	-ve
HI28	76 F	49 d	+	+++	+	-ve	++++	-ve	-ve	-ve	-ve

Case	Age/ sex	Survival period	Clusterin		βAPP		CD68*	Tau	Aβ	TDP43	p62
			Axons	Glial	Axons	Glial					
HI29	41 M	6 mo	-ve	+++	-ve	-ve	+++++	-ve	-ve	-ve	-ve
HI30 §	22 M	10 mo	-ve	+++	-ve	-ve	+++++	-ve	-ve	-ve	-ve
HI31	10 M	9 mo	-ve	+++	-ve	-ve	+++++	-ve	-ve	-ve	-ve
HI32 §	35 M	7 mo	-ve	++	-ve	-ve	+++++	-ve	-ve	-ve	-ve

Table 1: Case details and neuropathological results for Head Injury cases

+ occasional (1-2 per area), ++ few (3-6 per area), +++ many (7-10 per area), ++++ several (11-15 per area), +++++ numerous (more than 16 per area) -ve –No staining identified

CD68*: The quantification of CD68 is compared with control cases, which usually show a base line number of microglial cells. The number of pluses reflect, + slight, ++ more than slight, +++ moderate with microglial nodules, ++++ marked with microglial nodules and +++++ very marked increase of microglial cells with many microglial nodules.

§-*Also used in double immunofluorescence studies*

F-Female, M-Male, min-minutes, h-hours, d-days, mo-months

Number	Brain swelling	Contusions	SAH	SDH or EDH	Small haemorrhage in corpus callosum	Haemorrhage in brainstem	Ischaemia	Diagnosis
HI1	-	+ frontal	+	-	+	-	+	TBI
HI2	-	-	-	-	-	-	-	TBI
HI3	-	-	-	-	-	-	-	TBI
HI4	-	+ frontal	+	-	+	-	-	TBI
HI5	-	+ fronto- temporal	+	-	+	+	-	TBI
HI6	-	+ fronto- temporal	+	-	+	-	-	TBI
HI7	-	-	-	-	-	+	-	TBI
HI8	-	-	+	-	+	+	-	TBI
HI9	-	-	-	-	+	-	-	TBI
HI10	-	-	-	-	+	-	-	TBI
HI11	-	+ frontal	+	-	-	-	+ (hippo)	TBI
HI12	-	+ frontal	-	-	+	-		TBI
HI13	+	+ frontal	+	-	-	-	+	TB
HI14	-	-	+	-	+	-	+ (hippo)	TBI
HI15	+	+ fronto- temporal	+	-	-	-	+ (cortex)	TBI
HI16	+	+ fronto- temporal	+	-	+	-	+ (cortex)	TBI
HI17	+	+ fronto- temporal	+	-	-	+	+ (cortex)	TBI
HI18	-	-	+	-	+	-	-	TBI
HI19	+	+ fronto temporal	+	-	-	+	+ (cortex)	TBI

Number	Brain swelling	Contusions	SAH	SDH or EDH	Small haemorrhage in corpus callosum	Haemorrhage in brainstem	Ischaemia	Diagnosis
HI20	-	+ fronto- temporal	+	-	-	+	+ (cortex)	TBI
HI21	+	+ frontal	+	+ SDH	-	-	+ (cortex)	TBI
HI22	-	-	+	-	+	-	+ (cortex)	TBI
HI23	+	+ frontal	+	+ SDH	-	-	+ (cortex)	TBI
HI24	+	+ fronto-temporal	+	+ EDH	-	-	+	TBI
HI25	+	+ frontal	+	+ SDH	+	-	+	TBI
HI26	+	-	-	-	+	-	+	TBI
HI27	+	-	+	+ SDH		-	+	TBI
HI28	-	-	+	IV bleed	+	-	+	TBI
HI29	-	-	+ (old)	+ old SDH	-	-	-	Old TBI
HI30	-	+ fronto-temporal	+ (old)	+ old SDH	-	-	-	Old TBI
HI31	-	-	+ (old)	+ old SDH	-	-	+ (cortex)	Old TBI
HI32	-	-	+ (old)	+ old SDH	-	-	-	Old TBI

Table 2 – Illustrating the co-existing focal cerebral pathology in the traumatic brain injury cases.

SAH-subarchnoid haemorrhage, EDH-extra dural haemorrhage, SDH, subdural haemorrhage, IV intraventricular, TBI- traumatic brain injury, Hippo-hippocampus

+ - mild (in SAH-thin film; EDH <20 mls; SDH < 30 mls,; swelling - mild cerebral sulcal narrowing- no herniation)

- Negative/not present

No	Age/ sex	Diagnosis/Cause of Death	Clusterin		βAPP	Tau	Aβ	TDP43	p62
			Axons	Glial					
AD1 §	86F	Alzheimer's Disease (Braak stage VI)	-ve	+++	+++	++++	++++	-ve	++
AD2 §	86F	Alzheimer's Disease (Braak stage V1)	-ve	++	+++	++++	++++	-ve	++
AD3 §	92M	Alzheimer's Disease (Braak stage V1)	-ve	+++	+++	++++	++++	-ve	++
AD4	72M	Alzheimer's Disease	-ve	+++	+++	++++	++++	-ve	++
Isch1	37M	Hypovolemic shock	+++	++	+++	-ve	-ve	-ve	-ve
Isch2	63M	Cardiac arrest	+++	++	+++	-ve	-ve	-ve	-ve
Isch3	31F	Intestinal bleeding	+++	++	++++	-ve	-ve	-ve	-ve
C1	47M	Subarachnoid haemorrhage	-ve	-ve	-ve	-ve	-ve	-ve	-ve
C2	28M	Hypovolemic shock	-ve	-ve	-ve	-ve	-ve	-ve	-ve
C3	54M	Subarachnoid haemorrhage	-ve	-ve	-ve	-ve	-ve	-ve	-ve
C4	24M	Cardiac arrest	-ve	-ve	-ve	-ve	-ve	-ve	-ve
C5	36M	Sudden unexplained death – no evidence of brain pathology	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Table 3: Case details and neuropathological results from Alzheimer's disease, ischaemia and control cases.

For Clusterin, βAPP (Isch cases) : + occasional (1-2 per area), ++ few (3-6 per area), +++ many (7-10 per area), ++++ several (11-15 per area), +++++ numerous (more than 16 per area) -ve –No staining identified

For βAPP (AD cases), Tau, Aβ, TDP-43 and p62: +- minimal positivity, ++ few/mild, +++ moderate, ++++ severe/extensive positivity, -ve –No staining identified

§- *Also used for double immunofluorescence studies*

Primary Antibody	Type	Manufacturer	Dilution
Clusterin (ApoJ)	Polyclonal	AbCam	1:500
Clusterin (ApoJ)	Monoclonal	Santa Cruz	1:500
βAPP	Monoclonal	Chemicon	1,1000
GFAP	Polyclonal	DAKO	1:2000
CD68	Monoclonal	DAKO	1:50
HLA-DR (CR3/4)	Monoclonal	DAKO	1:100

Table 4: details of antibodies used in double immunofluorescence protocols.

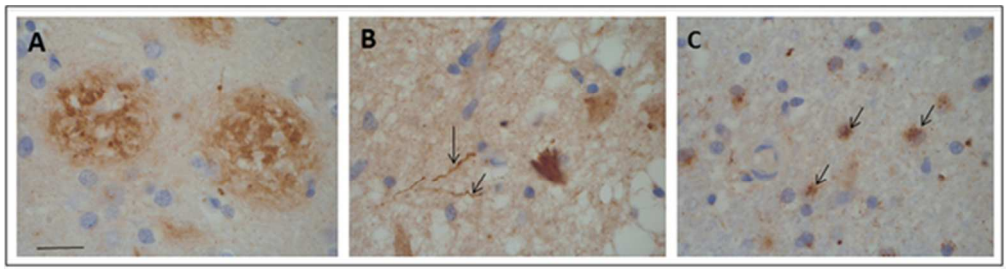

Figure 1: Clusterin expression in Alzheimer's disease cerebral tissue. Immunohistochemistry demonstrates Clusterin within neuritic plaques (A), neuropil threads (arrowed - B) and glial cells (arrowed - C). Scale bar represents 20μm.
52x13mm (300 x 300 DPI)

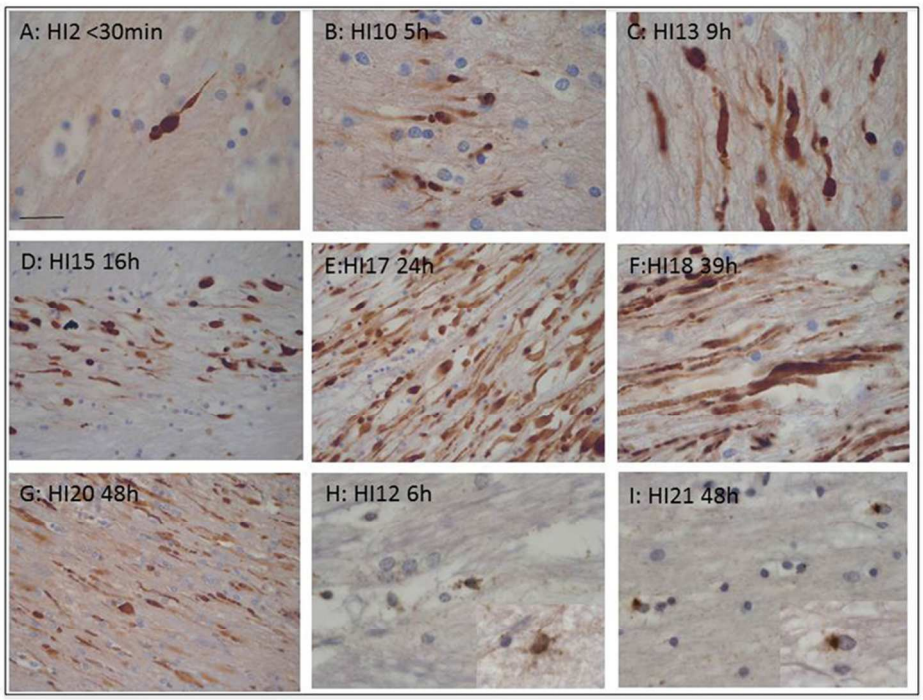

Figure 2: Clusterin expression shown in traumatic brain injury (TBI) cases with increasing time of survival from initial head injury. Clusterin is first seen to deposit as axonal globules, becoming most visible after several hours as globules and filaments. After 24 hours filaments are still numerous but intensity lessens. Glial expression becomes more prominent as survival time increases (up to 48 hours). (panels H & I show glial expression), insets show glial cells at higher magnification. Scale bar represents 20μm (A-C, F, H, I) and 50μm (D, E, G) and 15μm (H inset and I inset).
74x56mm (300 x 300 DPI)

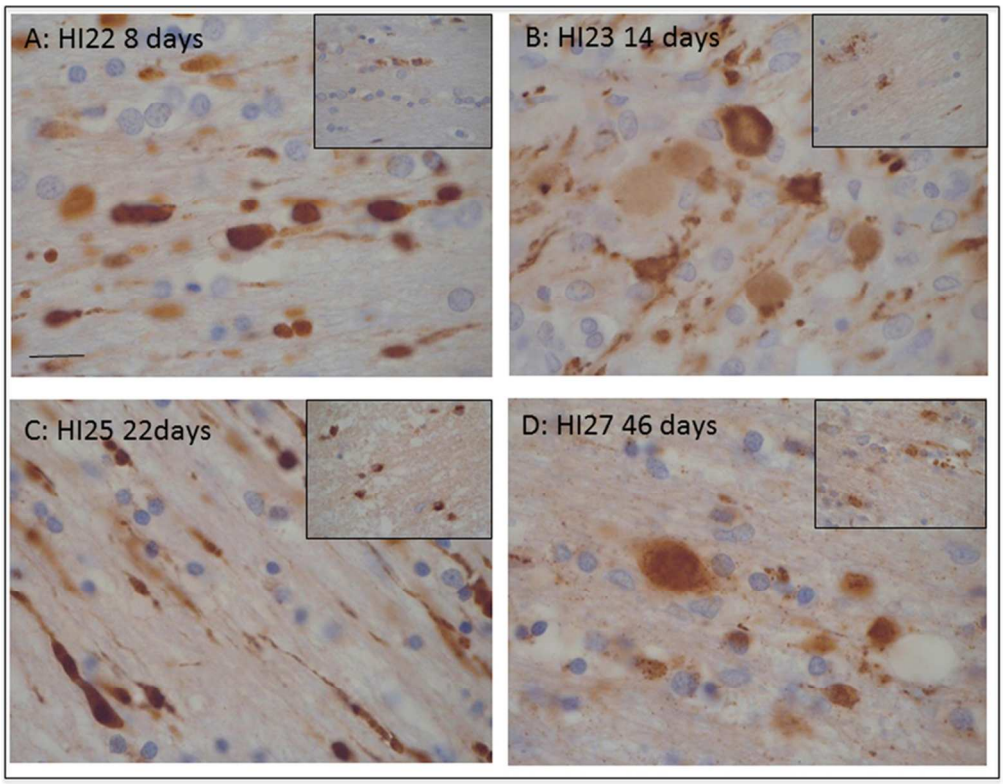

Figure 3: Clusterin expression demonstrated in TBI cases with a survival time of several days. Inset panels show glial expression. Scale bar represents 20μm (main images) and 50μm (insets).
77x60mm (300 x 300 DPI)

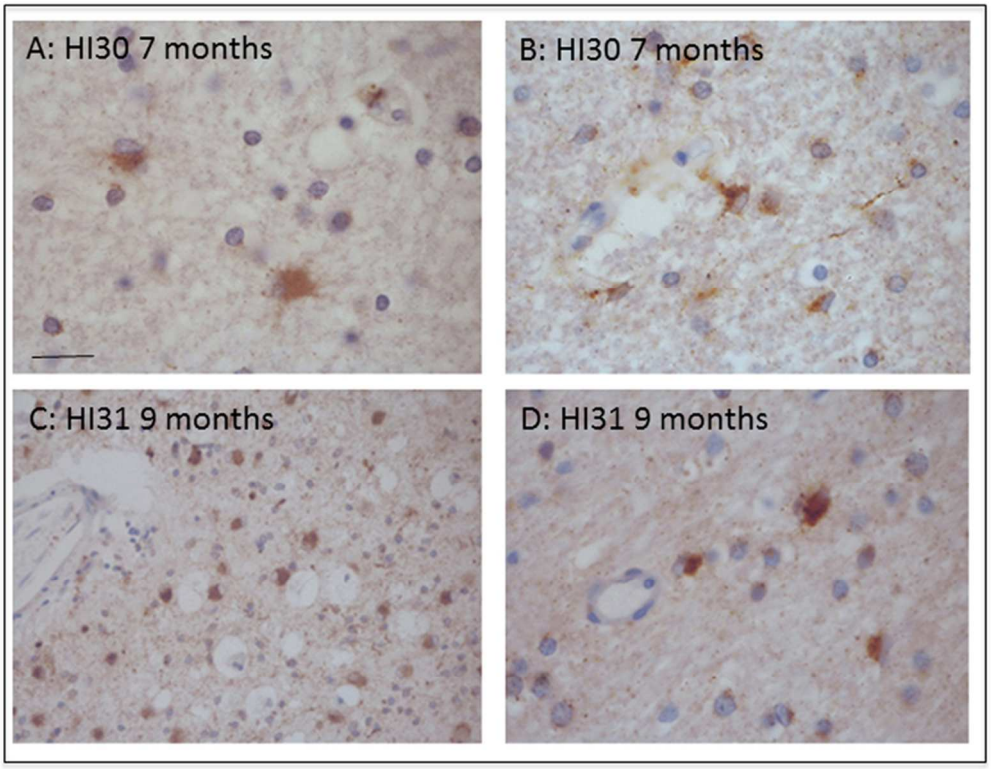

Figure 4: Clusterin expression demonstrated in old head injury cases with a survival time of over 6 months. Strong glial cell expression as cytoplasmic deposits can be seen within the white matter. Scale bar represents 20μm (A, B, D) and 50μm (C).
77x60mm (300 x 300 DPI)

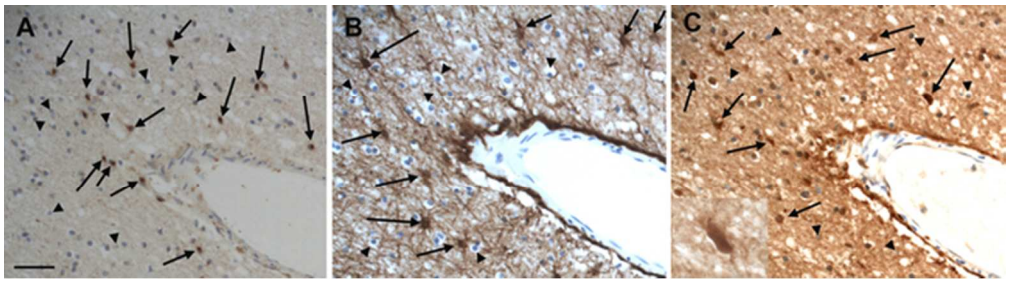

Figure 5: Adjacent sections from white matter in a case of old head injury with a survival of 9 months and showing the cellular immunoexpression of clusterin (A), GFAP (B) and S100 (C and inset). Note the cells expressing clusterin (arrows) appear to be very similar in shape and size to those reactive astrocytes positive for GFAP (arrows), and those astrocytes strongly expressing S100 (arrows), the smaller rounded oligodendrocytes (arrowheads) not staining for clusterin or GFAP and weaker for S100. Scale bar represents 50 µm (A-C), and 15µm (C inset).
54x14mm (300 x 300 DPI)

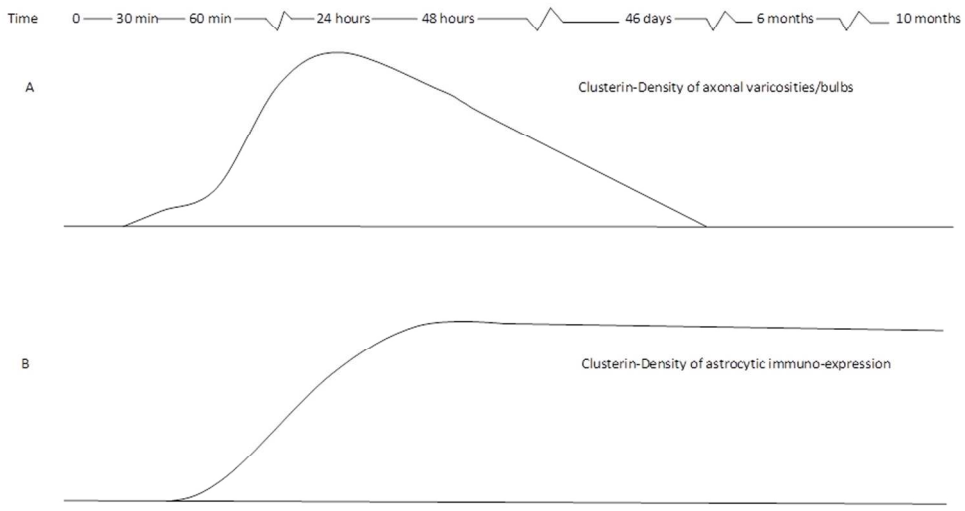

Figure 6: Schematic diagram illustrating the time line after survival from head injury and the relative immuno-expression of clusterin in axons (A) and astrocytic cells (B).
150x112mm (300 x 300 DPI)

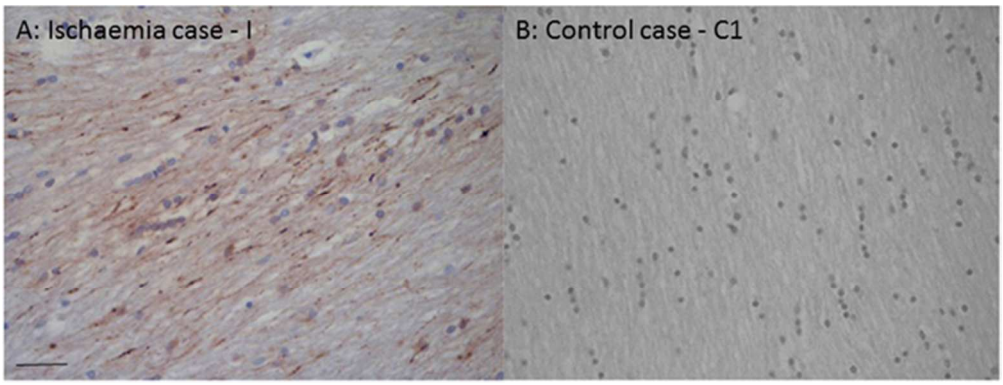

Figure 7: Clusterin expression in an ischaemia case, showing a granular and fine filamentous pattern (A) (white matter of the frontal lobe) and in a control case, showing no visible expression (B). Scale bar represents 50μm.
56x21mm (300 x 300 DPI)

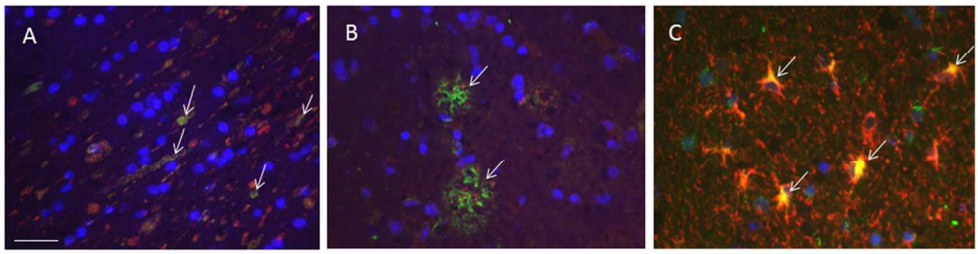

Figure 8: (A) Double immunofluorescence of clusterin (red) and βAPP (green) within an acute head injury case demonstrates areas of adjacent expression (indicated with arrows) as well as deposits of clusterin and βAPP alone. (B) Expression of both clusterin and βAPP within plaques can be seen in an AD case (arrows). (C) There is , co-localisation of clusterin and GFAP within astrocytes (arrows) in an old head injury case, but this is not evident in acute head injury. Scale bar represents 30μm.
112x84mm (300 x 300 DPI)

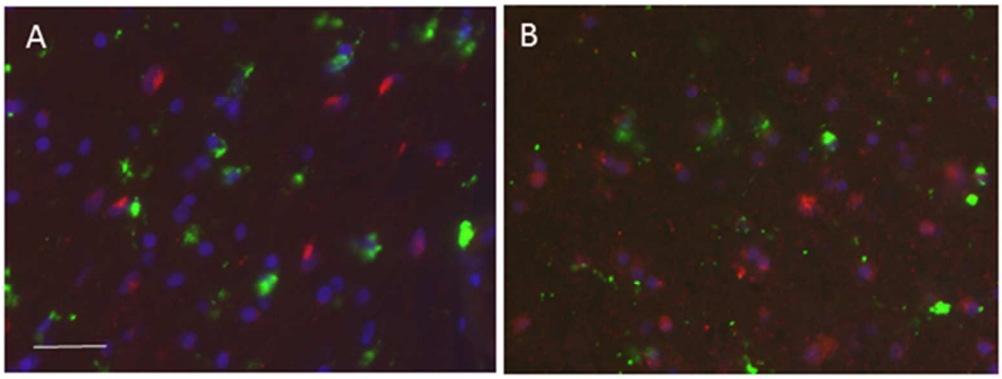

Figure 9: A and B: There is no co-localisation of clusterin (red), and microglia (CD68 - green) in any of the cases, however, cases of old head injury (A) showed somewhat increased microglial activation compared to acute head injury (B). Scale bar represents 30μm.
55x20mm (300 x 300 DPI)